PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 09 December 1999 (09.12.99)	
International application No. PCT/GB99/01234	**Applicant's or agent's file reference** 8.67972/002
International filing date (day/month/year) 22 April 1999 (22.04.99)	**Priority date** (day/month/year) 22 April 1998 (22.04.98)
Applicant BALINOV, Balin et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

22 November 1999 (22.11.99)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

Olivia RANAIVOJAONA

Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

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From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

MARSDEN, John, Christopher
Frank B. Dehn & Co.
179 Queen Victoria Street
London EC4V 4EL
ROYAUME-UNI

Date of mailing (day/month/year) 30 October 2000 (30.10.00)	
Applicant's or agent's file reference 8.67972/002	**IMPORTANT NOTIFICATION**
International application No. PCT/GB99/01234	**International filing date** (day/month/year) 22 April 1999 (22.04.99)

1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

Name and Address	State of Nationality	State of Residence
MARSDEN, John, Christopher Frank B. Dehn & Co. 179 Queen Victoria Street London EC4V 4EL United Kingdom	GB	GB
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[X] the person [X] the name [X] the address [ ] the nationality [ ] the residence

Name and Address	State of Nationality	State of Residence
NYCOMED IMAGING AS Nycoveien 2 P.O. Box 4220 Torshov N-0401 Oslo Norway	~~GB~~ NO	~~GB~~ NO
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

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3. Further observations, if necessary:
The person in Box 1 has assigned his rights to the person in Box 2.

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[ ] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Christine Carrié
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
8.67972/002		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB 99/01234	22/04/1999	22/04/1998
Applicant		
MARSDEN, John, Christopher et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 5 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 99/01234

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 18-20
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 18-20 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.: 1-22 in part
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
See FURTHER INFORMATION SHEET PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-22 in part

Present claims 1-22 relate to an extremely large number of possible agents, methods and uses. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the agents, methods and uses claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the agents, methods and uses for which pharmacological data were supplied, those mentioned specifically in the claims, and to the principle underlying the present application.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/01234

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K49/00 A61K41/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 25097 A (APFEL ENTERPRISES INC) 17 July 1997 (1997-07-17) abstract page 6, line 9 -page 7, line 23 page 32, line 12 -page 33, line 10 ---	1-22
X	WO 94 21301 A (HOLMES MICHAEL JOHN ;NYCOMED IMAGING AS (NO); BERG ARNE (NO); DUGS) 29 September 1994 (1994-09-29) abstract page 9, line 29 - line 37 ---	1-22
X	US 4 681 119 A (RASOR JULIA S ET AL) 21 July 1987 (1987-07-21) column 6, line 1 - line 24 column 8, line 59 -column 9, line 24 --- -/--	1-22

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 September 1999	06/10/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Dullaart, A

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4 466 442 A (ZIMMERMANN INGFRIED ET AL) 21 August 1984 (1984-08-21) column 4, line 63 -column 5, line 2 ---	1-22
A	SIMONIN J -P: "On the mechanisms of in vitro and in vivo phonophoresis" JOURNAL OF CONTROLLED RELEASE, vol. 33, no. 1, 1 January 1995 (1995-01-01), page 125-141 XP004037648 ISSN: 0168-3659 page 133 ---	1-22
P,X	WO 98 17324 A (MARSDEN JOHN CHRISTOPHER ;ERIKSEN MORTEN (NO); OESTENSEN JONNY (NO) 30 April 1998 (1998-04-30) page 57 -page 58; examples 1BW,1CA-1CC page 69; examples 2AO-2AQ -----	1-22

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 99/01234

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9725097 A	17-07-1997	AU 2241897 A	01-08-1997
		CA 2242768 A	17-07-1997
		EP 0892645 A	27-01-1999
		US 5840276 A	24-11-1998
WO 9421301 A	29-09-1994	AU 696091 B	03-09-1998
		AU 6215294 A	11-10-1994
		BG 62084 B	26-02-1999
		BG 100272 A	31-05-1996
		BR 9406228 A	12-12-1995
		CA 2158365 A	29-09-1994
		CN 1121315 A	24-04-1996
		CZ 9502370 A	13-03-1996
		EP 0689461 A	03-01-1996
		FI 954325 A	11-10-1995
		HU 72982 A	28-06-1996
		JP 8509706 T	15-10-1996
		NO 953637 A	15-09-1995
		PL 310656 A	27-12-1995
		SK 113895 A	05-02-1997
US 4681119 A	21-07-1987	AT 17311 T	15-01-1986
		AU 565842 B	01-10-1987
		AU 4127485 A	19-09-1985
		AU 545866 B	01-08-1985
		AU 7931382 A	07-06-1982
		CA 1170569 A	10-07-1984
		EP 0052575 A	26-05-1982
		WO 8201642 A	27-05-1982
		US 4442843 A	17-04-1984
		US 4657756 A	14-04-1987
US 4466442 A	21-08-1984	DE 3141641 A	28-04-1983
		AT 18356 T	15-03-1986
		AU 558152 B	22-01-1987
		AU 8916382 A	21-04-1983
		CA 1199577 A	21-01-1986
		DK 455782 A,B,	17-04-1983
		EP 0077752 A	27-04-1983
		FI 823474 A,B,	17-04-1983
		IE 55051 B	09-05-1990
		JP 2040803 C	09-04-1996
		JP 4043889 B	20-07-1992
		JP 58079930 A	13-05-1983
		NZ 202186 A	11-07-1986
		ZA 8207577 A	31-08-1983
WO 9817324 A	30-04-1998	AU 4714797 A	15-05-1998
		NO 991869 A	02-06-1999

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
8.67972/002		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB99/01234	22/04/1999	22/04/1998
International Patent Classification (IPC) or national classification and IPC A61K49/00		
Applicant MARSDEN, John, Christopher et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 10 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 1 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☒ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
22/11/1999	05.09.2000
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Baumgärtner, H Telephone No. +49 89 2399 8480

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB99/01234

I. Basis of th r port

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-20	as originally filed				
Claims, No.:					
7-22	as originally filed				
1-6	as received on	03/08/2000	with letter of	02/08/2000	

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 18-20.

because:

☒ the said international application, or the said claims Nos. 18-20 relate to the following subject matt r which does not require an international pr liminary examination (*specify*):

see separate sheet

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	
	No:	Claims	1-12, 15-17
Inventive step (IS)	Yes:	Claims	
	No:	Claims	13-14
Industrial applicability (IA)	Yes:	Claims	1-17 for claims 18-22 s. separate sheet
	No:	Claims	

2. Citations and explanations

see separate sheet

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Re Item III

Non-establishment of opinion with respect to novelty, inventive step and industrial applicability

Claims 18-20 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

The documents which are referred to in this communication are numbered in the order of their listing in the International Search Report.

D1: WO 97 25097 A (APFEL ENTERPRISES INC) 17 July 1997 (1997-07-17)
Activatable infusible dispersions and methods for using them for therapy and diagnostics
Claim 1 of D1 discloses the major features of the present application:
a dispersion for therapeutic or diagnostic use comprising an **aqueous continuous phase** suitable **for infusion** into a human [...] **dispersed drops** comprising a practically **immiscible superheated liquid** [...] permit their in-body **nucleation** by a level of [...] **ultrasound** [...].
The drop material is sufficiently superheated. The degree of superheat is very high [...] near but **at least a few degrees below** the **amount** of superheat **that causes homogeneous nucleation** (p.6/l.19-26).

D2: WO 94 21301 A (HOLMES MICHAEL JOHN ;NYCOMED IMAGING AS (NO); BERG ARNE (NO); DUGS) 29 September 1994 (1994-09-29)
refers to **contrast agents** of use in **diagnostic ultrasound imaging**.
The objective of the application may be fulfilled by oil-in-water emulsion- based contrast agents containing **oil-soluble gases/fluids or gas precursors** in condensed or dissolved form **in the dispersed oil phase** (p.2/l.19-23). On page 9/l.29 seq. it is outlined that the **oil phase** of contrast agents **may contain** suspended solid microparticles, which may act as **nucleation sit s** promoting generation of gas [...].

D3: US-A-4 681 119 (RASOR JULIA S ET AL) 21 July 1987 (1987-07-21)
D4: US-A-4 466 442 (ZIMMERMANN INGFRIED ET AL) 21 August 1984 (1984-08-21)
D5: WO 98 17324 A (MARSDEN JOHN CHRISTOPHER ;ERIKSEN MORTEN (NO); OESTENSEN JONNY (NO) 30 April 1998 (1998-04-30)

[D6: SIMONIN J -P: 'On the mechanisms of in vitro and in vivo phonophoresis' JOURNAL OF CONTROLLED RELEASE, vol. 33, no. 1, 1 January 1995 (1995-01-01), page 125-141 XP004037648 ISSN: 0168-3659]

Novelty(i), Inventive Step(ii) and Industrial Applicability (iii) - Art. 33 (1)-(4)

i.

Novelty of **claim 1** referring to an **ultrasound contrast agent comprising an injectable O/W emulsion wherein there are *heterogenous (cf. Item VIII)* gas-containing nucleation sites associated with the droplets of the dispersed oil phase** and of the thereon dependent claims 2-12 is considered to be **destroyed by D1**.

For depended claims s. below:	D1
dep 2) nucleation sites within dispersed oil phase droplets - (cf. Re. Item VIII)	p.6/l.9-19ff
dep 3) nucleations sites comprise	p.6/l.8-p.9/l.9-10
encaps	p.16/l.10-p.27/l.21
dep 4) nucleations sites within membranes/in contact w. outs.	p.8/l.19-21
dep 5) oil phase - ...aliphatic hydrocarbons...	p.5/l.24-25
dep 6) perfluorocarbons	dto
dep 7) perfluoro-butane/-pentane/hexane	dto.
dep. 8) oil phase ...gaseous solute	p. 20 bottom-p.21 top
dep. 9) -" - : air, oxygen etc.	p.21 top oxygen/p.23
dep.10) oil phase droplets stabilised by surfactants	p. 6/l.5ff
dep. 11) -"- by polymeric wall-forming encaps. material	p. 9/p.16 and 27
dep. 12) oil phase boiling point ≤ 42°C	p.7/l.8 below 20°C

Claims 15-17 related to a **drug delivery agent (accord. to cl. 1-12) plus a therapeutic drug** is **not novel** over the prior art, e.g. D1 where Mitomycin C is the therapeutic agent.

Claim 18 a **method of generating enhanced images of human/non-human subjects which comprises ... injecting a contrast agent (accord. 1-14) in to the vascular system and generating an ultrasound image** is equally **not novel** (cf. object of D1).

	D1
dep.19) microbubble growth form contrast agent	p.10/l.16
dep.20) external activation e.g. ultrasound	p.4/l.4-5-p.10/l.9-p.17

Claim 21 the **use of a contrast agent (accord. 1-12) in ultrasound therapy** is again **not novel** (cf. object of D1).

	D1
dep.22) therapy involves cell killing/blocking of blood flow	p. 23

ii.

The problem underlying the invention of claims 13 and 14 is to provide a further contrast agent with improved ultrasonic imaging properties.
The subject-matter of those claims differs from D1 by the addition of a vasodilatator drug. This is considered to have been obvious. It is of common practise in diagnostic procedures to dilate vessels with the agents proposed, particularly adenosine as a short-acting agent.

iii.

For the assessment of the present claims 18-22 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Re Item VI

Certain documents cited

Document D6 is presently not taken into account when assessing on novelty and inventive step as the priority documents were not available in due time. D6 might, however, become relevant in case the claimed priority cannot be maintained.

Certain published documents

Application No Patent No	Publication date *(day/month/year)*	Filing date *(day/month/year)*	Priority date *(valid claim)* *(day/month/year)*
PCT/GB97/02898	30.04.98	21.10.97	21.10.96

Re Item VIII

Certain observations on the international application

The term "***heterogeneous*** gas-containing ***nucleation sites***" is considered to be vague and thus not clearly being delimitable over the prior art (Art. 6 PCT). The ultrasound contrast agent composition as claimed and as described in the specification does not elucidate the claimed feature in a way which would allow the skilled person to clearly see the differences vis-à-vis the prior art.

The specification mentions "***gas-containing heterogeneous nucleation sites***" on page 4 without any further explanation.

specification — on p. 8/l.29 - p.9/l.13 it is stipulated that "***nucleation sites***" may be present within the dispersed oil phase droplets etc... , take the form of dispersed gas microbubbles, gas-containing porous solid micro particles...etc.
on p.5/l.1 et seq. it reads as follows: ...the ***agent comprises*** an ***injectable oil-in-water emulsion*** wherein there are ***gas containing nucleation sites associated with droplets of the dispersed oil phase.***

D1 — as mentioned above discloses ***drop material*** that is dispersed in an aqueous liquid (p.12/l.6-7). The preferred embodiment refers to a dispersion comprising an emulsifier, i.e. an ***emulsion*** (p.12/l.13-14). Methods for how to make the superheated drop dispersion include e.g.:

- 2) on p.14/l.6 ff; liquid drop material is dispersed (***oil-in-water dispersion***) into an aqueous intravenous fluid. The superheated drop dispersions are physically stable from premature vaporization (p.15/l.18-20).
- 3) on p.17/l. 8 ff; following infusion the drops are ***nucleated locally*** ... The energy source can take several form, e.g. ***x-ray, radiation, ultrasound*** etc.
The exact preparation procedure is described in detail in the example of p. 24.

--> Looking at the embodiment in e.g. example 4 b) of the application, where a perfluoroalkane is added to the hollow polymer-stabilised nanocapsules no indication from the mere preparational process can be found why the particles of D1 in comparison should not equally consist of nucleation sites similar to those of the application.

Apart from the novelty objection (s.supra) it should be noted that it is unclear why the Applicant describes the ***D1 nucleation as a "homogeneous nucleation"*** (which is by the way not to be found in D1. The only reference made on p.6/l.19ff - cf. also supra Item V - reads as follow: "most preferably the degree of superheat is [...], near but at least a few degrees below the amount of superheat that causes homogeneous nucleation", thus D1 appears to envisage an heterogeneous nucleation as well) and the nucleation of the ***application "heterogenous"***. In the description it is not further specified wherein the alleged differentiation is to be found.

D2 p. 9/l.29ff "the oil phase of contrast agents .... may additionally contain suspended ***solid microparticles*** ..... which ***may act as nucleation sites [...]. Such microparticles, which may for example comprise silica or iron oxide, may...***"
The contrast agents do consist of
p.11/l.1-23 and p.3/l.l.7ff: .... a solution of an oil-soluble gas/fluid or ***gas precursor*** (e.g. perfluoroalkane...) in at least one lipophilic solvent medium may be emulsified in an aqueous phase so as to form an ***oil-in-water-emulsion***

---> D2 ***seems with respect to nucleation sites to disclose the same subject-matter*** as the application, where the nucleation sites may be associated with the droplets of the oil phase.

specification cf. for instance Example 1 and 2: they let assume that Kaolin and Zeolithe do represent nucleation sites comparable to those of D2, but there is no clear evidence.

Claims

1. An ultrasound contrast agent comprising an injectable oil-in-water emulsion wherein there are heterogeneous gas-containing nucleation sites associated with droplets of the dispersed oil phase.

2. A contrast agent as claimed in claim 1 wherein the nucleation sites are present within dispersed oil phase droplets.

3. A contrast agent as claimed in claim 2 wherein the nucleation sites comprise free gas microbubbles, surfactant- or lipid-stabilised gas microbubbles, polymer- or protein-encapsulated gas microbubbles, gas-containing porous solid microparticles, gas-containing rough-surfaced solid microparticles, gas-containing polymeric microparticles, or gas-containing fullerenes, clathrates or nanotubes.

4. A contrast agent as claimed in claim 1 wherein nucleation sites are present within membranes stabilising the dispersed oil phase droplets or in contact with the outside of such membranes.

5. A contrast agent as claimed in any of the preceding claims wherein the oil phase comprises one or more components selected from aliphatic ethers, polycyclic oils and alcohols, heterocyclic compounds, aliphatic hydrocarbons, cycloaliphatic hydrocarbons and halogenated hydrocarbons, said component(s) having a boiling point not exceeding 60°C.

6. A contrast agent as claimed in claim 5 wherein the oil phase comprises one or more perfluorocarbons.

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(57) Abstract

Ultrasound contrast agents of the phase shift colloid type, comprising emulsions of volatile oils in water, are provided with gas–containing nucleation sites associated with (e.g. within) droplets of the dispersed oil phase, in order to enhance efficacy and control of the liquid–to–gas phase transition.

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Improvements in or relating to contrast agents

This invention relates to ultrasound imaging, more particularly to novel contrast agent preparations and their use in ultrasound imaging, for example in visualising tissue perfusion.

It is well known that contrast agents comprising dispersions of microbubbles of gases are particularly efficient backscatterers of ultrasound by virtue of the low density and ease of compressibility of the microbubbles. Such microbubble dispersions, if appropriately stabilised, may permit highly effective ultrasound visualisation of, for example, the vascular system and tissue microvasculature, often at advantageously low doses.

The use of ultrasonography to assess blood perfusion (i.e. blood flow per unit of tissue mass) is of potential value in, for example, tumour detection, tumour tissue typically having different vascularity from healthy tissue, and studies of the myocardium, e.g. to detect myocardial infarctions. A problem with the application of existing ultrasound contrast agents to cardiac perfusion studies is that the information content of images obtained is degraded by attenuation caused by contrast agent present in the ventricles of the heart.

In our copending International Patent Publication No. WO-A-9817324, the contents of which are incorporated herein by reference, we have disclosed that ultrasonic visualisation of a subject, in particular of perfusion in the myocardium and other tissues, may be achieved and/or enhanced by means of gas-containing contrast agent preparations which promote controllable and temporary growth of the gas phase *in vivo* following administration. Such contrast agent preparations may be

used to promote controllable and temporary retention of the gas phase, for example in the form of microbubbles, in tissue microvasculature, thereby enhancing the concentration of gas in such tissue and accordingly enhancing its echogenicity, e.g. relative to the blood pool.

Such use of gas as a deposited perfusion tracer differs markedly from existing proposals regarding intravenously administrable microbubble ultrasound contrast agents. Thus it is generally thought necessary to avoid microbubble growth since, if uncontrolled, this may lead to potentially hazardous tissue embolisation. Accordingly it may be necessary to limit the dose administered and/or to use gas mixtures with compositions selected so as to minimise bubble growth *in vivo* by inhibiting inward diffusion of blood gases into the microbubbles (see e.g. WO-A-9503835 and WO-A-9516467).

In accordance with WO-A-9817324, on the other hand, a composition comprising a dispersed gas phase is coadministered with a composition comprising at least one substance which has or is capable of generating a gas or vapour pressure *in vivo* sufficient to promote controllable growth of the said dispersed gas phase through inward diffusion thereto of molecules of gas or vapour derived from said substance, which for brevity is hereinafter referred to as a "diffusible component", although it will be appreciated that transport mechanisms other than diffusion may additionally or alternatively be involved in operation of the invention, as discussed in greater detail hereinafter.

This coadministration of a dispersed gas phase-containing composition and a composition comprising a diffusible component having an appropriate degree of volatility may be contrasted with previous proposals regarding administration of volatile substances alone, e.g. in the form of phase shift colloids as described in

WO-A-9416739. Thus the contrast agent preparations of WO-A-9817324 permit control of factors such as the probability and/or rate of growth of the dispersed gas by selection of appropriate constituents of the coadministered compositions, whereas administration of the aforementioned phase shift colloids alone may lead to generation of microbubbles which grow uncontrollably and unevenly, possibly to the extent where at least a proportion of the microbubbles may cause potentially dangerous embolisation of, for example, the myocardial vasculature and brain (see e.g. Schwarz, *Advances in Echo-Contrast [1994(3)]*, pp. 48-49).

It has been found that administration of phase shift colloids alone may not lead to reliable or consistent *in vivo* volatilisation of the dispersed phase to generate gas or vapour microbubbles. Grayburn *et al.* in *J. Am. Coll. Cardiol. 26(5) [1995]*, pp. 1340-1347 suggest that preactivation of perfluoropentane emulsions may be required to achieve myocardial opacification in dogs at effective imaging doses low enough to avoid haemodynamic side effects. An activation technique for such colloidal dispersions, involving application of hypobaric forces thereto, is described in WO-A-9640282; typically this involves partially filling a syringe with the emulsion and subsequently forcibly withdrawing and then releasing the plunger of the syringe to generate a transient pressure change which causes formation of gas microbubbles within the emulsion. This is an inherently somewhat cumbersome technique which may fail to give consistent levels of activation.

Again with regard to phase shift colloids, it is stated in US-A-5536489 that emulsions of water-insoluble gas-forming chemicals such as perfluoropentane may be used as contrast agents for site-specific imaging, the emulsions only generating a significant number of image-enhancing gas microbubbles upon application of ultrasonic energy to a specific location in the body

which it is desired to image. Our own research has shown, however, that emulsions of volatile compounds such as 2-methylbutane or perfluoropentane give no detectable echo enhancement either *in vitro* or *in vivo* when ultrasonicated at energy levels which are sufficient to give pronounced contrast effects using two component contrast agents in accordance with WO-A-9817324.

WO-A-9725097 discloses the administration of aqueous dispersions of superheated droplets of water-immiscible liquids which may be vaporised *in vivo* under the influence of radiation or ultrasound, which are said to induce homogeneous nucleation of the droplets. The dispersions may be used, *inter alia*, to form diagnostic contrast agents or selectively to deliver drugs to a localised body region.

The present invention is based on the finding that volatile emulsions of the phase shift colloid type in which gas-containing heterogeneous nucleation sites are associated with the emulsion droplets possess a number of valuable advantages. In particular, they permit perfusion imaging to be carried out in similar manner to that described in WO-A-9817324, but without the need to administer two separate compositions, thereby facilitating handling of the products. Moreover, factors such as the ultimate size of the gas microbubbles generated by the volatile dispersed phase may be controlled through parameters such as the droplet size of the emulsion and the nature and location of the nucleation sites which may readily be set during manufacture of the contrast agent. Thus the high yield of liquid-to-gas phase transition resulting from the presence of nucleation sites make it possible accurately to forecast the size of the formed microbubbles, so permitting controlled retention with a high safety profile.

Thus according to one aspect of the present

invention there is provided an ultrasound contrast agent comprising an injectable oil-in-water emulsion wherein there are gas-containing nucleation sites associated with droplets of the dispersed oil phase.

The invention further provides a method of generating enhanced images of a human or non-human animal subject which comprises the steps of injecting a contrast agent as defined above into the vascular system of said subject and generating an ultrasound image of at least a part of said subject.

The dispersed oil phase may comprise one or more appropriately volatile components where at least one component is at least partially insoluble in and immiscible with water. This component or mixture of components is advantageously a liquid at processing and storage temperature, which may for example be as low as -10°C if the aqueous phase contains appropriate antifreeze material, while being a gas or exhibiting sufficient vapour pressure, e.g. at least 50 mm Hg, preferably at least 100 mm Hg, at body temperature. Other less volatile substantially water-insoluble and water-immiscible components may if desired also be present in the oil phase.

Appropriate volatile components may, for example, be selected from the various lists of emulsifiable low boiling liquids given in the aforementioned WO-A-9416739, the contents of which are incorporated herein by reference. Specific examples of emulsifiable oil phase components include aliphatic ethers such as diethyl ether; polycyclic oils or alcohols such as menthol, camphor or eucalyptol; heterocyclic compounds such as furan or dioxane; aliphatic hydrocarbons, which may be saturated or unsaturated and straight chained or branched, e.g. as in n-butane, n-pentane, 2-methylpropane, 2-methylbutane, 2,2-dimethylpropane, 2,2-dimethylbutane, 2,3-dimethylbutane, 1-butene, 2-butene, 2-methylpropene, 1,2-butadiene, 1,3-butadiene, 2-methyl-

1-butene, 2-methyl-2-butene, isoprene, 1-pentene, 1,3-pentadiene, 1,4-pentadiene, butenyne, 1-butyne, 2-butyne or 1,3-butadiyne; cycloaliphatic hydrocarbons such as cyclobutane, cyclobutene, methylcyclopropane or cyclopentane; and halogenated low molecular weight hydrocarbons (e.g. containing up to 7 carbon atoms). Representative halogenated hydrocarbons include dichloromethane, methyl bromide, 1,2-dichloroethylene, 1,1-dichloroethane, 1-bromoethylene, 1-chloroethylene, ethyl bromide, ethyl chloride, 1-chloropropene, 3-chloropropene, 1-chloropropane, 2-chloropropane and t-butyl chloride. Advantageously at least some of the halogen atoms are fluorine atoms, for example as in dichlorofluoromethane, trichlorofluoromethane, 1,2-dichloro-1,2-difluoroethane, 1,2-dichloro-1,1,2,2-tetrafluoroethane, 1,1,2-trichloro-1,2,2-trifluoroethane, 2-bromo-2-chloro-1,1,1-trifluoroethane, 2-chloro-1,1,2-trifluoroethyl difluoromethyl ether, 1-chloro-2,2,2-trifluoroethyl difluoromethyl ether, partially fluorinated alkanes (e.g. pentafluoropropanes such as 1H,1H,3H-pentafluoropropane, hexafluorobutanes, nonafluorobutanes such as 2H-nonafluoro-*t*-butane, and decafluoropentanes such as 2H,3H-decafluoropentane), partially fluorinated alkenes (e.g. heptafluoropentenes such as 1H,1H,2H-heptafluoropent-1-ene, and nonafluorohexenes such as 1H,1H,2H-nonafluorohex-1-ene), fluorinated ethers (e.g. 2,2,3,3,3-pentafluoropropyl methyl ether or 2,2,3,3,3-pentafluoropropyl difluoromethyl ether) and, more preferably, perfluorocarbons. Examples of perfluorocarbons include perfluoroalkanes such as perfluorobutanes, perfluoropentanes, perfluorohexanes (e.g. perfluoro-2-methylpentane), perfluoroheptanes, perfluorooctanes, perfluorononanes and perfluorodecanes; perfluorocycloalkanes such as perfluorocyclobutane, perfluorodimethyl-cyclobutanes, perfluorocyclopentane and perfluoromethylcyclopentane; perfluoroalkenes such

as perfluorobutenes (e.g. perfluorobut-2-ene or perfluorobuta-1,3-diene), perfluoropentenes (e.g. perfluoropent-1-ene) and perfluorohexenes (e.g. perfluoro-2-methylpent-2-ene or perfluoro-4-methylpent-2-ene); perfluorocycloalkenes such as perfluorocyclopentene or perfluorocyclopentadiene; and perfluorinated alcohols such as perfluoro-*t*-butanol.

Such at least partially water-insoluble/immiscible volatile substances may contain dissolved materials which significantly increase the vapour pressure of the mixture. Such solute materials include gases such as air; nitrogen; oxygen; carbon dioxide; hydrogen; inert gases such as helium, argon, xenon or krypton; sulphur fluorides such as sulphur hexafluoride, disulphur decafluoride or trifluoromethylsulphur pentafluoride; selenium hexafluoride; optionally halogenated silanes such as methylsilane or dimethylsilane; low molecular weight hydrocarbons (e.g. containing up to 7 carbon atoms), for example alkanes such as methane, ethane, a propane, a butane or a pentane, cycloalkanes such as cyclopropane, cyclobutane or cyclopentane, alkenes such as ethylene, propene, propadiene or a butene, or alkynes such as acetylene or propyne; ethers such as dimethyl ether; ketones; esters; halogenated low molecular weight hydrocarbons (e.g. containing up to 7 carbon atoms); or mixtures of any of the foregoing. Gases such as air, oxygen and carbon dioxide, which have substantial solubility in fluorocarbon liquids, are preferred.

The emulsion will typically be stabilized by one or more surfactants or other encapsulating material. It will be appreciated that the nature of such material may significantly affect factors such as the rate of growth of volatilised gas. In general a wide range of surfactants may be useful, for example selected from the extensive lists given in EP-A-0727225, the contents of which are incorporated herein by reference. Representative examples of useful surfactants include

fatty acids (e.g. straight chain saturated or unsaturated fatty acids, for example containing 10-20 carbon atoms) and carbohydrate and triglyceride esters thereof, phospholipids (e.g. a lecithin or a fluorine-containing phospholipid), proteins (e.g. albumins such as human serum albumin), block copolymer surfactants (e.g. polyoxyethylene-polyoxypropylene block copolymers such as Pluronics, or extended polymers such as acyloxyacyl polyethylene glycols, for example polyethyleneglycol methyl ether 16-hexadecanoyloxy-hexadecanoate, e.g. wherein the polyethylene glycol moiety has a molecular weight of 2300, 5000 or 10000), fluorine-containing surfactants (e.g. as marketed under the trade names Zonyl and Fluorad, or as described in WO-A-9639197, the contents of which are incorporated herein by reference), and cationic surfactants, for example comprising one or more quaternary ammonium groups and one or more lipid groups such as long chain (e.g. C_{10-30}) alkyl or alkanoyl groups.

The emulsion droplets may also be stabilised by wall-forming encapsulating material, so that the dispersed phase is in the form of microcapsules containing the volatile liquid, or by incorporation into porous structures such as latex particles. Representative wall-forming materials include polymers such as polylactic acid, polycaprolactone, polycyanoacrylate and polyesters (e.g. as described in WO-A-9317718).

Nucleation sites may be present within the dispersed oil phase droplets or within surfactant or other encapsulating or stabilizing membranes surrounding the droplets; such membranes may themselves act as nucleation sites *per se*. Alternatively appropriate nucleation sites may be present in contact with the outside of such membranes.

Where the nucleation sites are present within the oil droplets they may, for example, take the form of

dispersed gas microbubbles, e.g. in the form of free microbubbles, surfactant- or lipid-stabilised microbubbles, polymer- or protein-encapsulated microbubbles, gas-containing porous solid microparticles such as aerogels or zeolites, gas entrapped in holes crevices or other irregularities of rough-surfaced solid microparticles, gas-containing polymeric microparticles or gas-containing entities such as fullerenes, clathrates or nanotubes. Such contrast agents may readily be prepared by dispersing the nucleation site-containing material in the oil phase and then generating an oil-in-water emulsion in *per se* known manner, using one or more appropriate dispersing agents.

In order to facilitate dispersion, the interfacial properties of nucleation sites may, for example, be varied by selection of a dispersing agent for the nucleation sites, or by chemical modification of the nucleation site surface, e.g. by silanisation or plasma modification. The presence of surface irregularities, cavities, edges, crevices or other structural defects which assist a gas phase in spreading on the interface may also be advantageous.

If desired, the nucleation sites may be selected to have interfacial properties which allow them to be located at the water-volatile oil interface. This may, for example, be achieved by choosing a dispersing agent for the nucleation sites which allows the surface of a nucleation site to be partly wetted by both the volatile oil and the aqueous phase. If necessary the surface of the nucleation site may be adjusted by chemical modification (e.g. plasma modification), rinsing etc.

In embodiments of the invention where it is desired that microbubble generation should occur spontaneously *in vivo*, it is generally preferred that the boiling point of the dispersed oil phase of the emulsion should not exceed 42°C, i.e that the sum of partial pressures from the volatile component(s) of the oil phase should

exceed one atmosphere at 42°C.

In other embodiments of the invention microbubbles may be generated either *in vivo* or immediately prior to injection by appropriate temperature and/or pressure modifications or application of external activating influences such as sound, ultrasound or radiation. When external activating influences are applied, emulsions in which the oil phase has a higher boiling point, e.g. up to 60°C, may also be useful, since the external activation may cause sufficient evaporation of the oil phase *in vivo* despite its boiling point being more removed from body temperature.

Microbubbles generated from contrast agents according to the present invention are characterised by a readily controllable rate of growth and final size; they may, for example be designed to grow to a size of e.g. 10-20 μm in order to exhibit controlled retention in tissue microvasculature, e.g. in the myocardium, or may be designed to grow to a size of e.g. 1-7 μm so that they behave as free-flowing contrast agents.

It will be appreciated that liquid-to-gas phase shift in emulsion droplets in the presence of nucleation sites ensures a highly efficient and rapid transformation of the liquid, hence limiting diffusion of volatile substance between separated particles and thus limiting uncontrolled bubble growth. In this respect, the material inside one emulsion droplet may be transformed to one bubble. Assuming a gas which can be described by the ideal gas law [Equation (1)],

$$pV = nRT \qquad (1)$$

where n is number of moles of substance to make one bubble and is related to the radius of the emulsion droplet, r_e, by Equation (2)

$$n = \frac{d \cdot V_e}{M_W} = \frac{d}{M_W} \cdot \frac{4}{3}\pi r_e^3 \qquad (2)$$

where d is the density of the liquid phase, M_W is the molecular weight of the volatile substance and V_e is the volume of the liquid droplet, then inserting Equation (2) into the ideal gas law Equation (1), and expressing the volume V of the obtained gas bubble by its radius r_b gives;

$$r_b = r_e \sqrt[3]{\frac{R\ T\ d}{p\ M_W}} \approx 0.29 \cdot r_e \sqrt[3]{\frac{d}{M_W}} \qquad (3)$$

For a typical volatile solvent, for example perfluoropentane, d is 1.66 g/ml, M_W = 288 g/mol and using T = 298 K and p = 1 atm, gives $r_b \approx 5.2 r_e$. The emulsion droplet should therefore have a size slightly below 2 μm in order to give a microbubble of size 10 μm which is therefore capable of temporary retention.

For the nucleation site to occupy 50% of such an emulsion droplet, its size should be below 1.6 μm. More preferably the nucleation site should occupy less than 20% of the emulsion droplet, so that its size should be below 1.2 μm; even more preferably, the nucleation site should occupy less than 10% of the liquid volume and so should have a size below 1 μm.

In order to ensure boiling of a sufficiently high number of emulsion droplets, a sufficiently high number of nucleation sites should be added. The nucleation sites will be distributed on the liquid carrier particles by simple Boltzmann distribution, and calculations may be made to estimate the amount of nucleation sites to be added for a given fraction of the liquid carrier particles to contain at least one nucleation site.

Activation of the phase transition from liquid to gas may be obtained by simply heating to temperatures above the boiling point of the volatile liquid. In order for phase transition to be activated on injection by utilizing the increase in temperature to body temperature, a volatile oil with boiling point below body temperature should be used. However, since bubble nucleation rate may be low also at elevated temperatures, the volatile liquid may have a boiling point well below body temperature. In such a superheated dispersion, presence of nucleation sites may lower the barrier for phase shift so that nucleation can be induced by means of an external influence.

Products in which gas formation is activated by ultrasonication or like treatment may be particularly advantageous in that they may be highly storage-stable prior to activation and use.

It will be appreciated that the dispersed gas content of contrast agents according to the invention will tend to be temporarily retained in tissue in concentrations proportional to the regional rate of tissue perfusion. Accordingly, when using ultrasound imaging modalities such as conventional or harmonic B-mode imaging where the display is derived directly from return signal intensities, images of such tissue may be interpreted as perfusion maps in which the displayed signal intensity is a function of local perfusion. This is in contrast to images obtained using free-flowing contrast agents, where the regional concentration of contrast agent and corresponding return signal intensity depend on the actual blood content rather than the rate of perfusion of local tissue.

In cardiac studies, where perfusion maps are derived from return signal intensities in accordance with this embodiment of the invention, it may be advantageous to subject a patient to physical or pharmacological stress in order to enhance the

distinction, and thus the difference in image intensities, between normally perfused myocardium and any myocardial regions supplied by stenotic arteries. As is known from radionuclide cardiac imaging, such stress induces vasodilatation and increased blood flow in healthy myocardial tissue, whereas blood flow in underperfused tissue supplied by a stenotic artery is substantially unchanged since the capacity for arteriolar vasodilatation is already exhausted by inherent autoregulation seeking to increase the restricted blood flow.

The application of stress as physical exercise or pharmacologically by administration of adrenergic agonists may cause discomfort such as chest pains in patient groups potentially suffering from heart disease, and it is therefore preferable to enhance the perfusion of healthy tissue by administration of a vasodilating drug, for example selected from adenosine, dipyridamole, nitroglycerine, isosorbide mononitrate, prazosin, doxazosin, dihydralazine, hydralazine, sodium nitroprusside, pentoxyphylline, amelodipine, felodipine, isradipine, nifedipine, nimodipine, verapamil, diltiazem and nitrous oxide. In the case of adenosine this may lead to in excess of fourfold increases in coronary blood flow in healthy myocardial tissue, greatly increasing the uptake and temporary retention of contrast agents in accordance with the invention and thus significantly increasing the difference in return signal intensities between normal and hypoperfused myocardial tissue. Because an essentially physical entrapment process is involved, retention of contrast agents according to the invention is highly efficient; this may be compared to the uptake of radionucleide tracers such as thallium 201 and technetium sestamibi, which is limited by low contact time between tracer and tissue and so may require maintenance of vasodilatation for the whole period of blood pool distribution for the

tracer (e.g. 4-6 minutes for thallium scintigraphy) to ensure optimum effect. The contrast agents of the invention, on the other hand, do not suffer such diffusion or transport limitations, and since their retention in myocardial tissue may also rapidly be terminated by the methods described above, the period of vasodilatation needed to achieve cardiac perfusion imaging in accordance with this embodiment of the invention may be very short, for example less than one minute. This will reduce the duration of any possible discomfort caused to patients by administration of vasodilator drugs.

In view of the fact that the required vasodilatation need only be short lasting, adenosine is a particularly useful vasodilating drug, being both an endogenous substance and having a very short-lasting action as evidenced by a blood pool half-life of only a few seconds. Vasodilatation will accordingly be most intense in the heart, since the drug will tend to reach more distal tissues in less than pharmacologically active concentrations. It will be appreciated that because of this short half-life, repeated injection or infusion of adenosine may be necessary during cardiac imaging in accordance with this embodiment of the invention; by way of example, an initial administration of 150 μg/kg of adenosine may be made substantially simultaneously with administration of the contrast agent composition, followed 10 seconds later by slow injection of a further 150 μg/kg of adenosine, e.g. over a period of 20 seconds.

The contrast agents of the invention may usefully be employed in therapeutic applications such as drug delivery agents. Thus hydrophobic drugs may be dissolved in the volatile oil phase to achieve an advantageously high drug load. Therapeutics may also be incorporated into any encapsulating membrane or may be dissolved in the aqueous carrier phase. Therapeutics

may also be present as nano- or micro-sized particles which may function as additional nucleation sites.

Without being bound with theoretical considerations, it is believed that evaporation of the volatile oil droplets will accelerate release of a dissolved therapeutic drug due to the increased concentration of drug in the liquid droplet, which may easily exceed the solubility level. Drug uptake may be also enhanced due to local shear and effects from "microstreaming" induced from the microbubble formation.

According to yet another aspect of the current invention, the induced liquid-to-gas transition may be utilised in applications such as ultrasound therapy. Thus, for example, the liquid-to-gas phase transition may provide microbubbles with a size sufficient to embolize capillaries, and hence may block blood flow to a site of interest, for instance a tumour, following appropriate application of localised ultrasound. The microbubbles may also absorb ultrasound energy and hence may provide heating of a site of interest which may be utilised in hyperthermia treatment. Furthermore, the liquid to gas transition may be very rapid, providing shear forces or microstreaming with a damaging effect on surrounding cells; this may be useful in cell killing, for example in treatment of cancer.

The following non-limitative Examples serve to illustrate the invention.

Example 1

A spatula edge of micronised kaolin is added to 2 ml perfluoropentane (b.p. 28°C) containing 0.2 ml Fluorad™ FC-171 surfactant. A milky white dispersion is obtained after gently shaking by hand. 0.1 ml of the above dispersion is mixed with 1 ml water by shaking on an Espe Capmix® for 30 seconds, yielding an emulsion with droplet size slightly above 1 μm.

A droplet of the emulsion is placed on a cooling/heating stage, and heated to 37°C while following the process in a microscope. Several 10 μm droplets appear, demonstrating a rapid liquid-to-gas phase shift in the emulsion droplets.

A tube containing the emulsion is dipped in a water bath maintained at 37°C so that only one part of the emulsion is heated. The turbidity immediately increases significantly in that part of the emulsion which is heated relatively to the non-heated emulsion, demonstrating the formation of small gas bubbles after heating.

Example 2

A spatula edge of micronised zeolite is added to 2 ml perfluoropentane (b.p. 28°C) containing 0.2 mg perfluorooctanoic acid. The sample is sonicated using a Branson W385 sonicator horn at 50% output power for two minutes while keeping the sample in an ice bath. 0.1 ml of the above dispersion is mixed with 1 ml water by shaking on an Espe Capmix® for 45 seconds, yielding an emulsion.

A sample of the emulsion (1 μl) is suspended in Isoton II (55 ml) at room temperature, and acoustic attenuation

is measured as a function of time using two broadband transducers with centre frequencies of 3.5 MHz and 5.0 MHz respectively, in a pulse-echo technique. The acoustic attenuation is weak. The sample is then heated step-wise and acoustic attenuation is measured for each temperature. When the sample temperature is around 30°C, a substantial increase in acoustic attenuation can be observed. This experiment demonstrates how a nucleation site-containing emulsion of a volatile substance can transform to a microbubble dispersion around its boiling point. It also demonstrates the change in acoustic properties and the product's usefulness as an ultrasound contrast agent.

Example 3 (comparative)

Example 2 is repeated without adding micronised zeolite to the perfluoropentane phase. When characterising the emulsion using the acoustic attenuation measurement technique, heating to temperatures well above 40°C leads only to a slight increase in acoustic attenuation. This demonstrates the requirement for nucleation sites to be associated with the dispersed phase.

Example 4

a) 5 ml of a 5% w/v solution of the polymer from Example 2(a) of WO-A-9607434 in (-)-camphene, maintained at 60°C, is added to 15 ml of a 5% w/v solution of human serum albumin in water at the same temperature. The mixture is mixed hot with an Ultra Turax T25 mixer at 20,000 rpm for 1 minute. Thereafter, the emulsion is homogenised at 60°C using an Emulsiflex C5 high-pressure homogeniser, operating at a peak pressure of 200,000 kPa and allowing five passes of the sample. The median size of the obtained emulsion is around 300 nm. The emulsion is then frozen on a dry ice/methanol bath and

lyophilised for 48 hours, giving a white powder. Electron microscopy indicates the formation of gas-filled nanocapsules. The polymer particles are dispersed in water and excess human serum albumin is removed by dialysis. The remaining polymer nanocapsules are dried under reduced pressure.

b) A spatula edge of the washed, hollow polymer-stabilised nanocapsules from (a) above is added to 2 ml perfluorodimethylcyclobutane (b.p. 45°C) containing 0.2 ml perfluorooctanoic acid. The sample is shaken on a laboratory shaker for one hour, yielding a dispersion of gas-filled nanocapsules dispersed in perfluorodimethylcyclobutane. 0.1 ml of the above dispersion is mixed with 1 ml water by shaking on an Espe Capmix® for 45 seconds, yielding an emulsion.

c) A droplet of the emulsion from (b) above is placed on a cooling/heating stage and heated to 50°C, while following the process in a microscope. Several 10 μm droplets appear when the temperature passes 45°C, demonstrating a rapid liquid-to-gas phase shift in the emulsion droplets.

d) A tube containing diluted emulsion from (b) above is dipped in a water bath maintained at 50°C, so that only part of the emulsion is heated. The turbidity immediately increases significantly in the heated part of the emulsion relative to the non-heated part, demonstrating the formation of small gas bubbles on heating.

e) A sample of the emulsion from (b) above (1 μl) is suspended in Isoton II (55 ml) at room temperature, and acoustic attenuation is measured as a function of time using two broadband transducers with centre frequencies of 3.5 MHz and 5.0 MHz respectively, in a pulse-echo

technique. The acoustic attenuation is weak. The sample is then heated step-wise and acoustic attenuation is measured for each temperature. When the sample temperature passes 35-40°C, a substantial increase in acoustic attenuation can be observed. This experiment demonstrates how a nucleation site-containing emulsion of a volatile substance can transform to a microbubble dispersion well below its boiling point when the emulsion is exposed to external ultrasound. It also demonstrates the change in acoustic properties and the product's usefulness as an ultrasound contrast agent.

Example 5

A dog is anaesthetised, a mid-line sternotomy is performed, and the anterior pericardium is removed. Mid-line short-axis B-mode imaging of the heart is performed through a low-attenuating 30 mm silicone rubber spacer, using an ATL HDI-3000 scanner equipped with a P3-2 transducer. The framerate is 40 Hz and the mechanical index is 1.1. An amount of the polymer nanocapsule-containing perfluorodimethylcyclobutane emulsion of Example 4(b), corresponding to 0.2 μl perfluorodimethylcyclobutane/kg body weight, is injected intravenously into the dog. A substantial rise in echo intensity from the myocardium is seen, starting some 20 seconds after the injection and lasting for 20 minutes. The increase in myocardial opacification is seen at a time when the ventricles are almost emptied of contrast. The observed efficacy is therefore due to microbubbles retarded in the myocardium.

Example 6 (comparative)

Example 5 is repeated except that a perfluorodimethyl-cyclobutane emulsion phase is used without added polymeric nanocapsules. *In vivo* ultrasound imaging

indicates limited acoustic efficacy of the emulsion. This comparative experiment shows the necessity for gas-filled nucleation site associated with the emulsion droplets.

Claims

1. An ultrasound contrast agent comprising an injectable oil-in-water emulsion wherein there are gas-containing nucleation sites associated with droplets of the dispersed oil phase.

2. A contrast agent as claimed in claim 1 wherein the nucleation sites are present within dispersed oil phase droplets.

3. A contrast agent as claimed in claim 2 wherein the nucleation sites comprise free gas microbubbles, surfactant- or lipid-stabilised gas microbubbles, polymer- or protein-encapsulated gas microbubbles, gas-containing porous solid microparticles, gas-containing rough-surfaced solid microparticles, gas-containing polymeric microparticles, or gas-containing fullerenes, clathrates or nanotubes.

4. A contrast agent as claimed in claim 1 wherein nucleation sites are present within membranes stabilising the dispersed oil phase droplets or in contact with the outside of such membranes.

5. A contrast agent as claimed in any of the preceding claims wherein the oil phase comprises one or more components selected from aliphatic ethers, polycyclic oils and alcohols, heterocyclic compounds, aliphatic hydrocarbons, cycloaliphatic hydrocarbons and halogenated hydrocarbons, said component(s) having a boiling point not exceeding 60°C.

6. A contrast agent as claimed in claim 5 wherein the oil phase comprises one or more perfluorocarbons.

7. A contrast agent as claimed in claim 6 wherein said perfluorocarbon is selected from perfluorobutanes, perfluoropentanes, perfluorohexanes, perfluorocyclobutane, perfluorodimethylcyclobutanes, perfluorocyclopentane, perfluoromethylcyclopentane, perfluorobutenes, perfluorobutadienes, perfluoropentenes, perfluorohexenes, perfluorocyclopentene, perfluorocyclopentadiene and perfluoro-*t*-butanol.

8. A contrast agent as claimed in any of the preceding claims wherein the oil phase contains a gaseous solute.

9. A contrast agent as claimed in claim 8 wherein the oil phase comprises air, oxygen or carbon dioxide dissolved in a liquid fluorocarbon.

10. A contrast agent as claimed in any of the preceding claims wherein the dispersed oil phase droplets are stabilised by a surfactant selected from fatty acids, carbohydrate and triglyceride esters of fatty acids, phospholipids, proteins, block copolymer surfactants, fluorine-containing surfactants and cationic surfactants.

11. A contrast agent as claimed in any of claims 1 to 9 wherein the dispersed oil phase droplets are stabilised by polymeric wall-forming encapsulating material or by incorporation into porous latex particles.

12. A contrast agent as claimed in any of the preceding claims wherein the oil phase has a boiling point not exceeding 42°C.

13. A combined preparation for simultaneous, separate or sequential use as a contrast agent in ultrasound imaging, said preparation comprising:

i) a contrast agent as claimed in any of the preceding claims, and

ii) a vasodilator drug.

14. A combined preparation as claimed in claim 13 wherein said vasodilator drug is adenosine.

15. A drug delivery agent comprising a contrast agent as claimed in any of claims 1 to 12 together with a therapeutic drug.

16. A drug delivery agent as claimed in claim 15 wherein a hydrophobic drug is dissolved in the oil phase.

17. A drug delivery agent as claimed in claim 15 wherein the drug is present as nano- or micro-sized particles.

18. A method of generating enhanced images of a human or non-human animal subject which comprises the steps of injecting a contrast agent as claimed in any of claims 1 to 14 into the vascular system of said subject and generating an ultrasound image of at least a part of said subject.

19. A method as claimed in claim 18 wherein microbubble growth from the contrast agent is activated within the subject by application of external activation.

20. A method as claimed in claim 19 wherein said external activation comprises ultrasound irradiation.

21. Use of a contrast agent as claimed in any of claims 1 to 12 in ultrasound therapy.

22. Use as claimed in claim 21 wherein said therapy involves cell killing or blocking of blood flow to a site of interest.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/01234

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K49/00 A61K41/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 25097 A (APFEL ENTERPRISES INC) 17 July 1997 (1997-07-17) abstract page 6, line 9 -page 7, line 23 page 32, line 12 -page 33, line 10 ---	1-22
X	WO 94 21301 A (HOLMES MICHAEL JOHN ;NYCOMED IMAGING AS (NO); BERG ARNE (NO); DUGS) 29 September 1994 (1994-09-29) abstract page 9, line 29 - line 37 ---	1-22
X	US 4 681 119 A (RASOR JULIA S ET AL) 21 July 1987 (1987-07-21) column 6, line 1 - line 24 column 8, line 59 -column 9, line 24 --- -/--	1-22

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 September 1999	06/10/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Dullaart, A

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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	US 4 466 442 A (ZIMMERMANN INGFRIED ET AL) 21 August 1984 (1984-08-21) column 4, line 63 -column 5, line 2	1-22
A	SIMONIN J -P: "On the mechanisms of in vitro and in vivo phonophoresis" JOURNAL OF CONTROLLED RELEASE, vol. 33, no. 1, 1 January 1995 (1995-01-01), page 125-141 XP004037648 ISSN: 0168-3659 page 133	1-22
P,X	WO 98 17324 A (MARSDEN JOHN CHRISTOPHER ;ERIKSEN MORTEN (NO); OESTENSEN JONNY (NO) 30 April 1998 (1998-04-30) page 57 -page 58; examples 1BW,1CA-1CC page 69; examples 2AO-2AQ	1-22

INTERNATIONAL SEARCH REPORT

nternational application No.
PCT/GB 99/01234

Box I Observations wher certain claims were found unsearchable (Continuati n of item 1 of first sh t)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 18-20
because they relate to subject matter not required to be searched by this Authority. namely:
Remark: Although claims 18-20 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.: 1-22 in part
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
See FURTHER INFORMATION SHEET PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee. this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant. this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently. this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-22 in part

Present claims 1-22 relate to an extremely large number of possible agents, methods and uses. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the agents, methods and uses claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the agents, methods and uses for which pharmacological data were supplied, those mentioned specifically in the claims, and to the principle underlying the present application.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 99/01234

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